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Modulation of Pathogenic B Cells through Inhibition of Phosphatidylinositol 3-Kinases

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Note: An abstract is required to be provided in Block 14

This proposal addresses the FY12 PRMRP topic area on lupus. Lupus is a life threatening disease that primarily affects women. Lupus patients develop antibodies that recognize proteins made by the body. This leads to tissue damage and complexes of the antibodies bound to the proteins can lodge in the kidneys resulting in damage to the filtering capacity of the kidney. The disease is most often managed using drugs that nonspecifically reduce inflammation and suppress the immune system. However that leaves the patient susceptible to other types of infections. Lupus treatment could be improved by specifically targeting the B cells involved in making the "self" antibodies. This proposal outlines one possible approach that could help solve this problem.

B cells express an important signaling molecule called PI3 kinase (PI3K). Activation of this enzyme leads to induction of survival pathways and is needed to promote development of antibodies. Therefore inhibition of PI3 kinase is expected to be beneficial for lupus by impairing survival of the pathogenic B cells and inhibiting their ability to produce antibodies. B cells express a specific form of PI3 kinase called delta. Small molecule inhibitors of the delta isoform have been shown to specifically kill B cell malignancies but leave other cells unaffected. Based on the success of the delta inhibitor in cancer research, it is anticipated that this approach will be particularly useful in lupus. Mice that are genetically predisposed to developing lupus will be treated with the PI3K delta inhibitor to determine if this ameliorates disease. If this works, it will provide an unprecedented level of control to target B cells and affect them by two mechanisms—survival and antibody production.

B cells also have survival mechanisms that work independently of PI3 kinase. One new lupus treatment is based on interfering with the other survival pathways. The drug, Benlysta, specifically neutralizes a B cell survival factor called BAFF (also known as Blys), which causes death of the mature antibody secreting B cells. Unfortunately it does not work for everyone and has shown little help among African-Americans. One possible reason is that the survival pathways mediated by PI3 kinase are capable to keeping many of the antibody producing B cells alive. Therefore, cultured B cells will be treated under conditions that mimic their interactions in the body to determine if interfering with both PI3K dependent and BAFF dependent survival results in even more B cell death than either approach alone. If true, experiments to test this in lupus prone animal models will be performed in the future.

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

   This project focuses on use of novel PI3kinase inhibitors to treat lupus. The PI3K/Akt pathway is a highly conserved pathway involved in numerous processes including survival. The immune system expresses a novel PI3K isoform referred to as delta, which plays a critical role in B cell signaling, antibody production, and survival. Recently PI3Kδ inhibitors have been developed to treat B cell malignancies. Since they target tumors that often have characteristics similar to antibody secreting B cells, we reasoned that a similar approach may be useful for treating lupus, a disease resulting in production of antibodies recognizes "self" components, such as nuclear proteins and DNA. These antibodies can cause additional pathologic changes because immune complexes lodge in the kidney which results in further damage. These experiments will test the hypothesis that PI3Kδ inhibition will reduce the frequency of pathogenic antibody (anti-dsDNA) secreting B cells in a mouse model for lupus, which results in less kidney damage and increased lifespan.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).
   Lupus, PI3K, B cell, signal transduction

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Progress Report for PI3K inhibitor on Lupus

Aim 1. Evaluate whether inhibition of PI3Kδ ameloriates the clinical pathology of lupus in NZB/NZW F1 mice

Aim 1. Our initial animal studies were designed to begin assessing the impact of treating lupus diseased mice with CAL101, a PI3Kδ inhibitor. Previous work by others showed that inhibition of PI3Kδ was a promising treatment for some classes of B cell malignancies. In fact, this class of inhibitors has been recently approved for mantle cell lymphoma. Tonic B cell receptor (BCR)

signaling is needed for B cell survival and BCR signaling in general leads to antibody and cytokine production. While B cells express multiple isoforms of PI3K, BCR signaling is highly dependent on delta isoform. We reasoned that inhibitors of PI3Kδ would lead to reduced antibody production and should decrease the number of activated B cells. This would be expected to reduce the amount of disease in lupus.

Overview

During the first year of this project, we established an approximate timeline when we could detect pathogenic anti-dsDNA antibodies in the NZB/NZW lupus mouse model. We also showed that a number of cellular hallmarks associated with lupus were reduced after 1 month of treatment with an inhibitor of PI3Kδ. Using flow cytometry, we showed reduced germinal center B cells (B cells that differentiate into antibody secreting cells) and Tfh (T cell subset that promotes germinal center B cells. Most of these results were summarized in the annual report for year 1. This promising data suggested that this treatment approach might be effective in controlling lupus.

During the period covered by this annual report (year 2), we extended the above studies to determine differences in survival between treated and untreated mice. The survival studies are ongoing and funded during the no cost extension portion of the project. We took monthly serum samples during the survival study to evaluate the levels of circulating pathogenic antibodies. These will be examined at the end of the survival study. During year 1 we stored spleens and kidneys from mice treated with drug for one month in order to perform histology during year 2. Kidneys are a site of damage caused by lupus; pathogenic antibodies get lodged in the glomeruli and elicit complement mediated lysis of the cells. The damage results in proteinurea. Histological sections from these tissues are then stained to determine if there were changes in splenic germinal centers and antibody deposition in the kidney.

Results

To mimic a therapeutic approach for evaluating the potential of blocking lupus with PI3Kδ inhibitors, we began dosing mice only after they showed elevated titers of anti-dsDNA antibodies. For the survival studies, four month NZB/NZW mice had increased levels of pathogenic antibodies and began receiving twice daily doses of 10 mg/kg CAL101 (idelalisib), a PI3Kδ inhibitor that was recently FDA approved for treating some types of B cell leukemias. Blood was collected after 2 weeks to measure IgG and anti-dsDNA Ab. After one month of dosing, a cohort of mice were euthanized and spleens, blood, bone marrow, and kidneys isolated for further analysis.

IgG serum titers. A typical mouse has serum IgG concentration of ~5 mg/ml. The NZB/NZW mice develop hypergammaglobulinemia as disease progresses. There was no discernable difference in IgG levels between vehicle and CAL101 after two weeks of dosing (Fig 1). In contrast, after 4 weeks of CAL101 treatment, the level of total IgG decreased and was statistically significant relative to vehicle alone. Since PI3Kδ inhibition has the potential of blocking all antibody production, we also examined IgG levels after 16 weeks on drug to determine whether the circulating antibody levels were compromised. Notably, the concentration of IgG did not differ from the 4 week samples. This is suggestive that the drug does not completely inhibit antibody production; rather longer term dosing reduces the level of

IgG back into a range considered normal for the mouse and may have a disproportionate effect on autoantibody production.

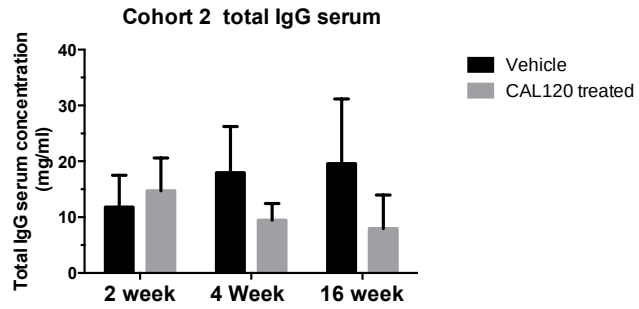

Fig 1. Reduction of hypergammaglobulinemia by treatment with PI3Kδ inhibitor. Mice began receiving inhibitor once IgG titers were increased over pre-symptomatic mice (~4 months). Total IgG decreased after only 4 weeks on drug and levels remained constant throughout over 16 weeks of dosing.

Anti-dsDNA titers. A hallmark of lupus is the production of autoantibodies against nuclear constituents, including dsDNA. We measured serum titers of anti-dsDNA in mice treated with either vehicle or the PI3Kδ inhibitor (Fig 2). After 4 weeks dosing with the PI3Kδ inhibitor, there was a marked decreased in these pathogenic antibodies, suggesting that this approach may be capable of managing the disease.

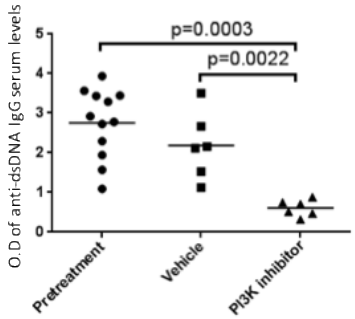

Fig 2. PI3Kδ inhibition lowers levels of circulating anti-dsDNA. Mice were initially screened for presence of anti-dsDNA antibodies. Animals with elevated levels were treated for 4 weeks with inhibitor then anti-dsDNA antibodies measured by ELISA. Levels dropped after 4 weeks.

Antibody deposition in kidney. Many patients with lupus develop lupus nephritis, which is caused by antibody complexes getting lodged in the kidney glomeruli. This allows for complement fixation, which results in damage to the glomeruli. Over time, the glomeruli no longer filter appropriately and the urine contains large amounts of protein. The level of proteinuria was variable in mice on the initial study. The subsequent study which will be presented in the final progress report shows that the NZB/NZW mice treated with vehicle develop proteinuria, while mice dosed with the PI3Kδ inhibitor have only trace levels, similar to non-lupus prone mice.

We also monitored IgG deposition in the kidneys (Fig 3). While most glomeruli from mice treated with vehicle showed Ig deposits, this was strongly reduced after only 4 weeks on the inhibitor. This result suggests the possibility of reversing or preventing further advancement of lupus nephritis. Ongoing studies are designed to determine if complement fixation is altered by treatment with the PI3Kδ inhibitor.

Splenic changes in NZB/NZW mice treated with PI3Kδ inhibitor. The NZB/NZW mice develop splenomegaly as disease progresses. This is accompanied by an increase in germinal centers, which are sites where B cells differentiate into antibody secreting cells. Over time, the

size of germinal centers also increases in the lupus prone mice. After 2 weeks on the inhibitor, the spleens showed a trend towards becoming smaller. The reduction in splenomegaly became apparent after 4 weeks on the drug and was statistically significant (Fig 4). We also examined the presence of germinal centers. In a presympomatic NZB/NZW mice, germinal centers are rare and small. In contrast, as disease progresses, the number of germinal centers increases markedly (Figs 5 and 6). The germinal centers are identified by PNA staining (green) and are located inside the B cell follicles (marked by IgD staining). In concert with the reduction in splenomegaly, the frequency of germinal centers also decreased by treatment with the PI3Kδ inhibitor and reached statistical significance after 4 weeks of treatment. Notably the number of B cell follicles was unaffected by the drug.

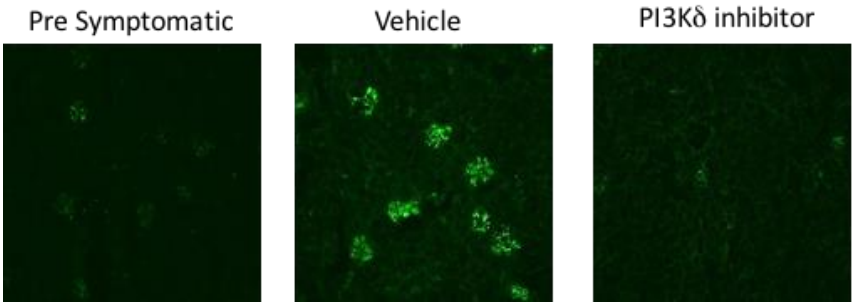

Fig 3. Glomerular immune complexes are reduced following treatment with PI3Kδ inhibitor. Kidneys were sectioned and stained with anti-IgG to detect IgG deposits. Notably Ig deposits are highly reduced after 4 weeks on inhibitor.
Magnification=100x.

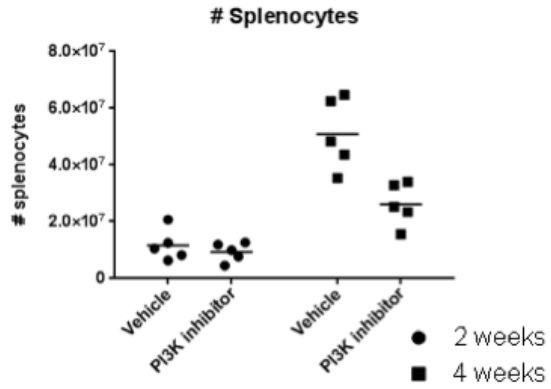

Fig 4. Splenocyte numbers are reduced in drug treated mice. As disease progresses the spleen enlarges. Splenomegaly is reduced during drug treatment. It becomes statistically significant after 4 weeks.

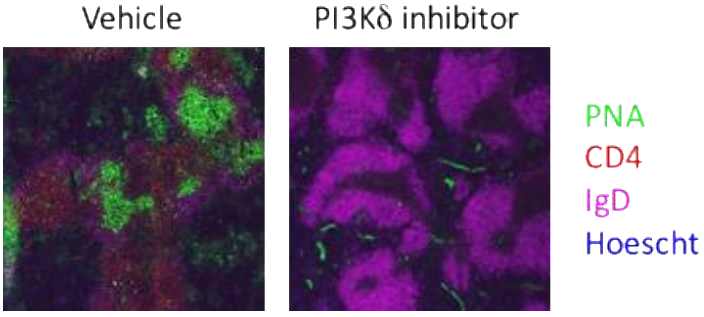

Fig 5. Germinal Centers (GC) are reduced after 4 weeks treatment with PI3Kd inhibitor. Spleens were sectioned and stained with markers indicated on panel. The reduction in GC is consistent with reduction in pathogenic antibodies. PNA: marks germinal centers; IgD: marks B cell follicles; CD4: marks CD4 T cells, possibly Tfh

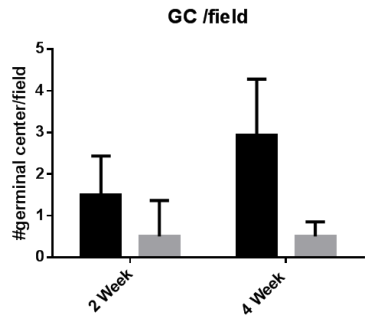

Fig 6. Quantitation of germinal centers (GC) in spleen. The number of PNA+ germinal centers (sites of antibody producing B cells) were counted in 10 fields from 5 spleens and average number of GC plotted. Germinal center frequency decreased after 2 weeks of treatment and remained reduced following 4 weeks on inhibitor. Black bar=vehicle treatment; grey bar=PI3Kδ treated mice

Aim 2. Determine if simultaneous inhibition of PI3K and BAFF synergistically impairs survival of pathogenic B cells.

Aim 2. These experiments were summarized in the annual report for year 1.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

Key Research Accomplishments:

- Treatment with the PI3Kδ inhibitor results in rapid reduction in hypergammaglobulinemia that is associated with lupus. Maximal response appears after 4 weeks of treatment
- Levels of pathogenic dsDNA antibodies highly reduced within 4 weeks of treatment
- Antibody deposits in kidney disappear following treatment. Suggestive that this treatment has potential to reverse kidney damage in lupus nephritis
- Splenomegaly and germinal centers (sites of B cells developing into antibody producing cells) in spleen reduced to levels observed in non-lupus prone mice

5. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The data accumulated during year 2 supports the preliminary conclusions drawn from year 1. Based on these results, treatment with the PI3Kδ inhibitor is expected to hold much promise for treating lupus. During as little as 4 weeks of treatment, virtually all the markers associated with lupus were reversed. We showed that the elevated amount of IgG and pathogenic anti-dsDNA antibodies were reduced to levels typically seen in non-lupus prone mice. While the survival arm of this study is still ongoing, the negligible level of proteinuria found in PI3Kδ inhibitor treated mice is consistent with the idea that this treatment prevents

lupus nephritis, a significant morbidity factor in humans. Consistent with this is the observation that Ig deposits in the kidney are cleared during treatment, thus sparing kidney function.

Since lupus is a chronic disease, the safety profile for new treatments must be very high since patients will be taking drugs for a long time. While the PI3Kδ inhibitors are approved for cancer treatment, there is more leeway in terms of acceptable safety. To limit exposure to the drug, one approach that might be worth considering is to dose mice for 4 weeks, then remove drug to determine how much time needs to elapse before there is an increase in pathogenic (anti-dsDNA) antibodies. If one round of treatment allows the mice to remain symptom-free for several months, treatment could be limited to a few times per year.

Targeting PI3Kδ is thought to predominantly affect the B cell compartment. However at least one report suggests that the delta isoform promotes Tfh development. Thus the efficacy of the inhibitor could be derived from the concerted effect on both the B cell and Tfh, which drives the germinal center response. It may be possible to dissect the relative contribution of the two cell types using more selective B cell inhibitors, such as those targeting Btk, a kinase needed for BCR signaling and B cell survival. Moreover, the selectivity of the Btk inhibitor (more B cell specific) may have fewer side effects which could be more permissive for use in chronic illnesses.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:
(2) Peer-Reviewed Scientific Journals:
(3) Invited Articles:
(4) Abstracts:
Nothing to Report

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
Nothing to Report

7. INVENTIONS, PATENTS AND LICENSES: List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.
Nothing to Report

8. REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution

toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

Nothing to Report

9. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Nothing to Report

For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

10. **REFERENCES:** N/A

11. **APPENDICES:** N/A

NOTE:

TRAINING OR FELLOWSHIP AWARDS: N/A

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A